METHOD FOR MEASURING NON-TRANSFERRIN BOUND IRON

Field of the Invention

The present invention relates to a method for measuring metal ions in biological fluids. More specifically, the invention relates to a method for the determination of iron in bodily fluids by measuring the level of non-transferrin-bound iron (NTBI).

Background of the Invention

The presence of non-transferrin-bound iron (NTBI) in the circulation is a pathological phenomenon which occurs in patients with iron-overload conditions. NTBI is absent from healthy individuals where virtually all of the serum iron is bound to the iron-carrier protein transferrin. However, in iron-overloaded individuals, the iron binding capacity of transferrin is overwhelmed, resulting in the adsorption of the excess iron to various proteins and possibly other molecules in the serum. This fraction is collectively designated as NTBI.

Generally, chronic iron-overload accompanied by NTBI occurs as a result of pathological conditions associated with specific diseases. Illustrative examples of such conditions include: 1) repeated transfusions, which are required by patients with various hemolytic diseases, hemoglobinopathies (among which the most common is thalassemia) or other forms of anemia whose treatment demands blood transfusions and/or infusion of iron *(e.g.* dialysis patients) and 2) an inherited defect causing excess iron absorption, called Hereditary Hemachromatosis. Transient, reversible NTBI can also appear in the

circulation of patients undergoing chemotherapy, heart bypass operations and other conditions where large amounts of iron, such as from hemoglobin catabolism, are suddenly released into the circulation. Recently, NTBI was also found in dialysis patients who are treated for anemia with erythropoietin and IV iron supplements (Breuer, Slotki and Cabantchik, unpublished).

The clinical significance of NTBI is threefold: 1. it is a primary target of iron-chelating drugs, which are used to remove excess body iron, 2. it is an indicator of the iron status of an individual who is already iron overloaded or is at risk and 3. it is thought to be one of the causative factors in the numerous and severe clinical consequences of chronic iron overload, which include cardiac, hepatic and endocrine disease. Unfortunately, the art has so far failed to provide a convenient and reliable method for detection and quantification of NTBI. As explained above, such a method would be very important in therapy: in the first case, it would allow the physician to assess the efficacy of an iron-overloaded patient's chelation regimen and modify it accordingly. In the second case, it could be used to help diagnose individuals who are suspected of being iron-overloaded. No less importantly, the availability of a rapid and inexpensive NTBI-test could open the possibility of screening high risk populations, since the genetic mutation causing Hereditary Hemachromatosis has a very high frequency (1 in 8) in Northern European and American populations and double that number in some African and African-American populations. Such an NTBI-screen might be particularly valuable for identifying those individuals with Hereditary Hemachromatosis who are initially misdiagnosed as they appear to be symptom-free in the first decades of life, with apparently normal transferrin-iron saturation levels. In those cases, low level NTBI might already be present

and detectable before the appearance of overt iron-overload related disease(s) which become manifest only later in life. Indeed, a recent newsletter of the "Iron-overload Diseases Association" has emphasized the need for such a test.

The Prior Art

Presently available diagnostic methods are limited in scope, and are basically divided into two groups:

***Detection of iron-overload*:**

Three routine clinical tests are available for detecting excess iron in the circulation: 1. total serum iron by chemical or physicochemical methods, 2. per cent transferrin-iron saturation, or serum iron-binding capacity, by measuring high-affinity binding of radioactive iron to serum components essentially transferrin) and 3. circulating ferritin levels by immunoassay. Although these three indicators tend to be elevated in most cases of severe iron-overload, they often fail to detect lower iron-load levels and can also fluctuate for reasons unrelated to iron-status. The most commonly used of these tests is for circulating ferritin levels, even though its diagnostic value for iron-status is controversial and can even be misleading in some cases. Since excess body iron accumulates first in the liver, analysis of liver biopsies constitutes a definitive diagnosis of iron-overload disease.

Detection of NTBI:

There are two main methods for NTBI determination currently used in research laboratories. However, because of their drawbacks, as explained below, they are not in routine clinical use.

One methodology was originally developed by Hershko and coworkers [Hershko, H., Graham, G., Bates, G.W., and Rachmilewitz (1978) ***British J. Haematol.*** **40, 255-263**] and later refined by Singh and coworkers [Singh, S., Hider, R.C. and Porter, J.B. (1990) ***Anal. Biochem.*** **186, 320-323**]. In brief, the refined method is as follows:

Step 1. A serum sample (1 ml) is mixed with 80 mM nitrilotriacetic acid (to solubilize the NTBI);

Step 2. The sample is filtered by centrifugation on Centricon filters with a 25 kD molecular weight cut-off;

Step 3. The protein-free filtrate is injected into an HPLC column derivatized with the iron chelator deferriprone (or L1), which forms a stoichiometric coloured complex with iron giving a quantitative value of the amount of iron in the sample.

The three main drawbacks of this method are its cost, its cumbersome nature, which makes it difficult to set up in non-specialized laboratories, and its relatively low throughput efficiency.

A second method [Evans, P.J. and Halliwell, B. (1994) ***Methods Enzymol.*, 233, 82-89**] employs the antibiotic bleomycin, which combines with NTBI, but not with transferrin-bound iron, to form highly reactive complexes which generate DNA cleavage products. The relative amount of DNA cleavage products is proportional to the amount of input NTBI and is quantified by the thiobarbituric acid test. The drawback of this method is that it tends to overestimate NTBI and may give false positive results.

It is an object of the present invention to provide a method for the measurement of NTBI in serum and in biological fluids, which overcomes all the drawbacks of prior art methods.

It is a further object of the invention to provide a method for measuring NTBI, which is reliable and convenient.

It is another object of the invention to provide conjugated functionalized polymers for use in the method of the invention, which are biologically compatible, reproducible, effective and inexpensive metal ion chelating substrates for coating of diagnostic plates for metal ion qualitative and quantitative detection.

It is still a further object of the invention to provide kits for the measurement of NTBI.

Other advantages and objects of the invention will become apparent as the description proceeds.

Summary of the Invention

The present invention provides a method for determining the concentration of a non-bound metal ion in a sample of serum or other biological fluids, comprising the steps of:

a) providing a surface coated with a polymer-conjugated form of a metal chelator;

b) bringing said sample into contact with said coated surface, under conditions and for a period of time sufficient to allow the metal ion to be captured by the metal chelator;

c) bringing into contact with said coated surface, after completion of step b) above, a marker conjugated with a moiety that can be captured by the metal chelator, which can be, e.g., the same metal ion the concentration of which it is desired to determine;

d) determining the amount of marker that has been released by the capture of the metal ion by the coated surface; and

e) calculating the concentration of the metal ion in the sample from the concentration of binding sites left available after step b) for capturing the metal ion bound to the marker.

By "marker" it is meant to indicate any substance the concentration of which can be precisely determined. This includes any form of visual, physical, electrical or chemical determination, such as fluorescent or chromogenic determination. The term "conjugated" in this context means any combination in which one end is suitable to combine with the bound chelator, and another end is detectable and functions as a marker. Such a combination may typically be a complex. According to a preferred embodiment of the

invention the marker is a calcein-iron complex. Another marker - an antibody which will bind specifically to the DFO-Fe complex (DFO - desferrioxamine) and will be quantified by ELISA assays.

According to a preferred embodiment of the invention the metal ion is non-transferrin bound iron (NTBI). According to another preferred embodiment of the invention the polymer-conjugated form of a metal chelator is a desferrioxamine (DFO) polymer.

The surface onto which the metal and the marker are bound can be of any suitable type and shape. Typically, the surface can be a multiwell plate of the type commonly employed, e.g., for fluorescent measurements.

While iron is the most important metal for determination in biological fluids, as explained above, the invention is in no way limited to the determination of non-bound iron, and can be used for determining other metals, e.g. aluminum (Al^{3+}).

The invention is further directed to the use of a polymer-conjugated form of a metal chelator for the determination of the concentration of a non-bound metal ion in a sample of serum or other biological fluids.

In another aspect, the invention is directed to a polymer for use in the determination of the concentration of a non-bound metal ion in a sample of serum or other biological fluids, characterized in that it is conjugated to a metal chelator, such as DFO or a DFO derivative.

Many different hydrophobic and hydrophilic polymers can be used. Illustrative and non-limitative examples of suitable polymers include polystyrene, polyethylene, polycarbonate, polyester polymers and copolymers, polysaccharides, acrylate-based poly(hydroxamic acid), and polypeptides containing hydroxamate groups.

When the chelator is DFO or a DFO derivative, it can be conjugated, for instance, to a polymer selected from polyacrylate, polyacrylate derivatives, polyacrylate copolymers, arabinogalactan, dextran, pollulan, cellulose and their derivatives.

The invention also encompasses a kit for the determination of the concentration of a non-bound metal ion in a sample of serum or other biological fluids, comprising a surface coated with a polymer-conjugated form of a metal chelator.

An illustrative kit of the invention may comprise a multiwell plate, coated with a polymer conjugated with DFO or a DFO derivative, such as polymeric arabinogalactan-DFO or hydroxyethyl starch-DFO conjugate. The kit may further comprise a marker conjugated with the same metal ion the concentration of which it is desired to determine.

It was surprisingly found that a class of water soluble DFO-conjugated polysaccharides which include the polysaccharide arabinogalactan-DFO and hydroxyethyl starch DFO as well as polypeptide DFO conjugates strongly adheres to commercial culture plastic trays

so that it can be used for the detection (diagnosis) of free iron ions in blood and other biological fluids and aqueous solutions.

Brief Description of the Drawings

In the drawings:

- Fig. 1 is a combined iron calibration curve from four separate tests, wherein the iron concentration is expressed in micromoles (μM) and the relative fluorescence in arbitrary units;
- Fig. 2 is a representative graph showing NTBI measurements for 4 normal and 3 thalassemic sera, and the direct relationship between the input volume of serum, expressed in microliters (μl) and the NTBI measurement expressed in fluorescence units; and
- Fig. 3 shows the binding of Al^{3+} to DFO-plate, expressed in fluorescence units, as a function of the Al concentration, expressed as micromoles (μM) in the presence and absence of normal serum.

Detailed Description of Preferred Embodiments

The NTBI test of the present invention has the following advantages:

1. It provides two distinct NTBI values- one is the NTBI which is **directly** accessible to the chelator DFO, the other is NTBI which is not directly DFO-accessible and requires "solubilization" or "mobilization" with nitrilotriacetate;

2. Technical simplicity and low labor intensity;

3. The results are obtained relatively quickly – the total testing time is approximately 5 hours, which includes 2 incubations of 2 hours each. Hundreds of samples can therefore be processed in parallel;

4. Hemoglobin found in occasionally hemolytic samples does not interfere with the assay, and

5. The reagents employed are all inexpensive and commercially available. The exception is the DFO-conjugated polymer which was produced either in the laboratory as Ara-Gal-DFO or was received in the form of hydroxyethyl-starch-DFO.

6. Overall low cost- the only sophisticated instrumentation necessary is a fluorescent plate reader, which is commonly available in laboratory settings;

7. No false positives- although earlier versions of the test gave NTBI values above zero with some healthy sera, the latest version has given negative values with all healthy sera tested so far (see Table 2, under "Tests using HES-DFO plates & $MnCl_2$ added").
Points 1,2,3,5 and 6 compare favorably with the NTBI test based on filtration/HPLC, while point 7 compares favorably with the test based on bleomycin.

The various steps of the invention will be briefly described below. Whenever reference is made to DFO or to a specific polymer, it is made with the understanding that the description of specific materials is provided for the purposes of illustration only, and that

any such description is not intended to limit the invention in any way. Other materials can be easily substituted by the skilled person, and any such substitution will of course be within the scope of the present invention.

GENERAL PROCEDURES

Step 1. Preparation of the Ara-Gal-desferrioxamine (DFO) coated multiwell plates (DFO-plates). In general, a solution of 0.5 mg/ml of a polysaccharide-desferrioxamine polymer (see below) or alternatively, hydroxyethyl starch-desferrioxamine (HES-DFO) polymer (obtained from Biomedical Frontiers Inc., Minnesota) is prepared in 10 mM sodium phosphate pH 8.6. From this solution 0.1 ml is transferred to each well in a 96-well plate. The F96 Maxisorp plates (NUNC, Denmark) have given satisfactory results. The plates are stored for 72 hr at 4°C, then washed x 3 in deionized water and stored dry in the cold until needed. Prior to use, the wells are blocked by incubation with 0.1 ml of 1% bovine serum albumin in phosphate buffered saline for 30 min. at 37°C and then washed x 2 with deionized water.

Step 1a. Preparation of DFO-conjugated polymers.

The preparation of DFO–polysaccharide conjugates comprises four steps (Sub-steps a-d):

a. Formation of active polysaccharide by oxidation with periodate.

b. Purification of an oxidized polysaccharide from interfering anions and by-products.

c. Coupling of desferrioxamine or an iron chelating agent to an oxidized polysaccharide by Schiff base formation and purification.

d. Reduction of the Schiff base to amine conjugate

Sub-Steps a-b. Oxidation of the polysaccharide. The reaction of periodate oxidation of a polysaccharide leading to oxidized polysaccharide (OP) is achieved by applying the reaction mixture to a column filled with a strongly basic anion-exchanger (Dowex-1, 2 or Amberlite) in the acetate form [obtained by the pre-treatment of the commercial anion exchanger (Dowex or Amberlite, obtained from Sigma) by 1M acetic acid solution]. This process of purification is fast, of high yield, high capacity, and the anion-exchanger could be easily regenerated.

Sub-Steps c-d. Preparation of Schiff base-conjugated desferrioxamine. OP forms Schiff bases with amino groups of desferrioxamine molecules. The coupling yield is a function of polymer molecular weight, desferrioxamine-polymer ratio, reaction pH, temperature and reaction time. Polysaccharides with a molecular weight range of 9,300 -74,000 reacted with desferrioxamine in aqueous buffer solutions of pH range between 7.5-10 at 4-37° C for 2-20 hrs to obtain up to 40% w/w desferrioxamine content the polymer product. The Schiff base bonds are converted into stable amine bonds by sodium or potassium borohydride.

Conjugates are separated from the salts (acetate, borate), low-molecular-weight polymer fractions (existed in the initial polymer and also formed as a result of partial degradation of OP) and the traces of the unbound water soluble drug by dialysis against de-ionized water for 24-48 hrs at 4°C using molecular porous membrane tubing followed by centrifugation and lyophilization.

Conjugation of metal ion chelating agents can be achieved by amidation or esterification of carboxyl, hydroxyl or amino group on the agent with the corresponding functional groups on the polymer or monomer.

DFO and other metal ion complexing agents can be conjugated to a polymeric carrier with other active groups along the polymer chain including carboxylic acids and its active derivatives (acid chloride, anhydride, active esters). An attractive activated polymer class is polymers containing anhydride bonds that are stable and easily reacting with amino and hydroxyl groups. For example, Poly (maleic anhydride-methyl vinyl ether) is a commercially available polymer of various molecular weights and compositions which was used for the conjugation of DFO (see Example 1).

Other maleic anhydride copolymers can be used as polymer carrier such as poly(maleic anhydride-octadecene) (available from Polysciences).

Other anhydride containing polymers that can substitute Poly(maleic anhydride-methyl vinyl ether) are copolymers of methacrylate anhydride with alkyl methacrylates, octadecene or other vinyl monomers. These anhydride derivatives are prepared from the reaction of methacrylic acid or acrylic acid copolymers with acetic anhydride at reflux for 30 min. and evaporation of the solvent and purification of a chloroformic solution in a mixture of etherpetroleum ether 1:1 v/v.

Alternatively, these anhydride containing polymers are coated on the plastic plates from an organic solution such as tetrahydrofuran, acetone and their mixtures with ethanol. The activated plastic plates are then reacted with an aqueous solution of DFO or any other amino containing dye for 10 hours at room temperature. The conjugation yield to the surface was more than 40% of the added dye.

To allow the conjugation of carboxylic acid containing metal ion complexing agents such as calcein, the anhydride activated plates were reacted with ethylene diamine or lysine to form amino groups on the surface available for amide conjugation with carboxylic acids. The agents are then conjugated via an amide bond using amidation catalysts such as 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI:HCl) in water solution.

Other amino containing polymers that can be used for conjugation or carboxylic acid containing agents or modified agents to contain a carboxylic acid (for example an hydroxyl or amino group on a complexing agent can be reacted with maleic or succinic anhydride to for form a half ester or half amide with a free carboxylic acid) include: polyamines that are hydrophilic insoluble or slightly soluble in aqueous solution but soluble in common organic solvents, preferably, alcohol or alcohol/water solutions. Examples of such polymers are: partially alkylated polyhethylenimine (PEI); partially alkylated poly(lysine) or polyurnitine; partially alkylated homo- and co-polymers of vinylamine or acrylate containing amino groups, and copolymers of vinylamine or acrylate containing amino groups with hydrophobic monomers. Typical examples are partially alkylated PEI with hydrophobic fatty chains that reduces its water solubility. The alkyl side groups encores the PEI to the hydrophobic surface of the plastic plate while allowing the PEI chains between encores to hydrate and interact with the cells.

A typical procedure for the alkylation of a polyamine is as follows: PEI of 30,000 molecular weight was partially alkylated with linear alkyl halide (chloride or bromide) with a chain length of 10 to 18 methylene groups. The degree of alkylation ranged from 1 to 10% of the amino groups of the PEI. The reaction between dry PEI and the alkyl halide was carried out in a dry organic solvent (toluene, chloroform or

dimethylformamide) at reflux for a few hours. The alkylation yield was determined by NMR analysis. The solubility of the polymer in alcohol and in water was determined. Alkylated PEI containing 2 to 5% stearly groups were insoluble in water but were soluble in alcohol. These PEI derivatives of molecular weights between 5,000 and 2,000,000 were used for cell culture plate coating.

DEP can be conjugated to a carboxylic acid containing polysaccharide via an amide bond. Natural polysaccharides containing hydroxyl side groups can be reacted with chloroacetic acid via an ether bond to form carboxylic acid side chains available for esterification or amidation. An example of such polymer is carboxymethyl cellulose. Calcein, another candidate for metal ion chelation was conjugated to a polysaccharide such as arabinogalactan and dextran by introducing first a primary amino group that can be then conjugated to an oxidized polysaccharide. In a typical example, Calcein was complexed with Cu^{+2} ions and then reacted with ethylene diamine in the presence of an amide conjugation catalyst, EDCI to form an amide bond with the benzoic carboxylic acid group on calcein. The modified amino containing calcein was then conjugated to oxidized AG or dextran to form an imine bond which was then hydrogenated to an amine bond.

Dimethyl ether 1:1 v/v mixture. The precipitated polymer contained at least 80% of the entry DFO to the reaction conjugated via an amide bond to the polymer. The precipitated polymer was then dissolved in acetone (50 ml) and 5 ml of water was added to hydrolyze the unreacted anhydride bonds and the polymer was precipitated in water. The precipitate was dissolved in acetone or methyl ethyl ketone or their mixtures with ethanol and

tetrahydrofuran and sprayed or added to the plastic plates at the desired amount and dried at room air to form a uniform coverage.

Step 2. Preparation of the serum sample. Each sample is subjected to two separate treatments: 1. To a serum sample of 0.1 ml is added 0.1 ml of 100 mM nitrilotriacetic acid, (Na-form, pH 7.4 containing 10 mM $MnCl_2$). The function of the nitrilotriacetic acid is to solubilize the NTBI, and of the Mn^{2+} to minimize iron binding by serum components. The sample may then be serially 2-fold diluted in 0.1 ml of 20 mM Hepes-Na, pH 7.3 containing 5 mM $MnCl_2$, or a single 1:12 dilution in the same dilution buffer may be prepared. 2. A second serum sample of 0.1 ml is mixed with 0.1 ml of 20 mM Hepes-Na, pH 7.3 and then serially 2-fold diluted in 20 mM Hepes-Na pH 7.3, or singly diluted in the same buffer, 1:12 . Iron standards for calibration are prepared by mixing 0.1 ml of 100 mM nitrilotriacetic acid, (Na-form, pH 7.4) containing 10 mM $MnCl_2$, with 0.1 ml of 20 ? M ferrous ammonium sulfate dissolved in 20 mM Hepes-Na, pH 7.3 buffer and serial dilution in the same dilution buffer.

Step 3. Transfer of the samples to DFO-plate and binding of NTBI to DFO. An aliquot of 0.1 ml is transferred from each serum sample dilution prepared in Step 2 into a well of the DFO-coated plate. The plate is incubated for 2 hrs at 37°C. The plate is then washed two times in deionized water, once in 5 mM EDTA pH 8.0 (to remove the Mn^{2+}) and again three times in deionized water.

Step 4. Addition of calcein-iron complex (cal-Fe) to the wells and fluorescence development. A stock solution of the **cal-Fe** complex is prepared by adding 4 μM ferrous

ammonium sulfate to 5 μM of the metal-sensitive, fluorescent probe calcein (**cal**) in 20 mM Hepes-Na, pH 7.3, followed by incubation at 37°C for >20 min. A 1: 25 dilution of the stock is prepared in 20 mM Hepes-Na, pH 7.3 to give 200nM cal/160 nM Fe (this solution is stable and can be stored), and 0.1 ml of this is added to each well. After 2 hrs at 37°C, the plate is read in a fluorescent plate reader with settings for Fluorescein (Ex 485/ Em 538).

Step 5. Calculation of the NTBI and contents. The fluorescence in the wells is determined in a multiwell plate reader (BMG LabTechnologies, Offenburg, Germany) with excitation/emission filters of 485/538 nm and gain of 25. The NTBI in each sample is calculated by comparing its fluorescence reading with the standard curve, and with appropriate compensation for the dilution. For generating the calibration curve, standard solutions containing known concentrations of iron ranging from 0.00125 to 400 μM were produced by serial 2-fold dilution (in 20 mM Hepes pH 7.2) of a starting solution containing 400 μM ferrous ammonium sulfate and 50 mM nitriloacetate. A 0.1 ml sample from each standard solution was added to DFO-coated wells, and the assay was carried out as described. The calibration curve is linear in the range 0.2 - 1 μM Fe (in 0.1 ml volume), and therefore a 1:12 final dilution of most serum samples will fall into this range. A combined iron calibration curve from four separate tests is shown in Fig. 1. Samples with NTBI <2 μM or >10 μM may require dilution adjustments. The use of nitrilotriacetate is necessary for solubilizing the NTBI, however this compound is slightly contaminated with iron, which may contribute to the assay. This is compensated by the use of nitrilotriacetate in the calibration.

The conditions, concentrations and amounts referred to in the general procedures described above are, of course, indicative. Different conditions, concentrations and amounts can be used in different cases, and the skilled person will easily adapt the above procedure to specific working conditions.

The invention will now be further illustrated with reference to the following examples of preferred embodiments thereof.

Example 1

Preparation of acrylate containing desferrioxamine polymers

To a polymerization kettle equipped with a stirrer and a nitrogen inlet there was added desferyl methacrylate (30g, prepared from the reaction of desferrioxamine with methacryloyl chloride), lauryl methacrylate (30g), benzoyl peroxide (0.5 g) and dry tetrahydrofuran (300ml). The kettle was immersed into a water bath at 60°C and allowed to stir over night. After cooling to room temperature the solvent was evaporated to about 100 ml, diethylether (500ml) was added and the white polymer precipitate was isolated by filtration and washed twice with diethyl ether and dried at room air. The polymer was soluble in organic solvents but insoluble in water. The polymer was used to coat plates.

Polymers of different ratio of these monomers have been prepared using a similar procedure. The results obtained were comparable to those obtained with the above polymer, and therefore they are not described herein in detail, for the sake of brevity. Other monomers such as alkylcrylate or methacrylate with alkyl chains of 5 to 20

methylene, dimethyl amino and aminoalkyl methacrylates or acrylate can also be used. Reference is made hereinafter to the preferred embodiments employing DFO attached to polymeric arabinogalactan (ArG-DFO) and hydroxyethyl starch.

Example 2

NTBI Testing

The sera of 15 patients with transfusional iron overload from Shaarei Tzedek Hospital in Jerusalem were analyzed. In Table 1 are presented results with 15 thalassemic sera, carried out on four different occasions and using three variations of the NTBI test. These variations are:

1. "Tests using ArG-DFO plates and no $MnCl_2$ added". In this test, the plastic multiwell plates were coated with the ArG-DFO polymer and the serum samples contained no added $MnCl_2$.

2. "Tests using ArG-DFO plates and $MnCl_2$ added". In this test, the same plastic multiwell plates coated with the ArG-DFO polymer were used. However, the serum samples contained 5 mM $MnCl_2$. The addition of Mn^{2+} was found to enhance the sensitivity of the test as evidenced by the higher NTBI values obtained (compared to variation 1., without $MnCl_2$). This occurs because the Mn^{2+} decreases the loss of iron due to non-specific adsorption to plastic and other sites. More importantly, Mn^{2+} competitively inhibits the high affinity binding of serum apo-transferrin to iron which was solubilized from the NTBI by the NTA (nitrilotriacetate).

3. "Tests using HES-DFO plates and $MnCl_2$ added". In this test, plastic multiwell plates coated with the HES-DFO polymer were used, and the serum samples contained 5 mM $MnCl_2$.

The critical common denominator in the results in Table 2 is that fourteen of the fifteen thalassemic patients have NTBI, with levels ranging from +0.4 to +11.6 μM regardless of when the serum samples were taken and of the variation in the test method. In contrast, as shown in Table 2, control, non-thalassemic sera have significantly lower values, ranging from -13.2 to +2.7 μM. The negative values are artifactual and stem from individual variability in non-iron related serum components. Therefore, all negative values are operationally defined as 0 μM NTBI. Also, the positive values obtained with some control sera are considered to be artifacts, which may have been related to the use of ArG-DFO plates. The basis for this conclusion comes from tests using HES-DFO plates, shown in Table 2 under the column "Tests using HES-DFO plates, & $MnCl_2$ added". With this method, all of the eleven control, non-thalassemic sera examined showed negative NTBI values (ranging from -13.2 to -1.7 μM), which are taken as 0 μM. For this reason, the current routine method employs HES-DFO plates and $MnCl_2$ added to serum.

Example 3

Some of the results of Tables 1 and 2 are shown in Fig. 2, which is a representative graph showing NTBI measurements for 4 normal sera (curves 1, 2, 3 and 4) and 3 thalassemic sera (curves 5, 6 and 7), and the direct relationship between the input volume of serum

and the NTBI measurement. The decrease in fluorescence is proportional to increasing NTBI concentration. Accordingly, the normal (control) sera show little or no decrease in fluorescence even at the highest sample volumes. The slight decrease observed at higher volumes is considered artifactual and is attributable to non-iron related serum components such as lipids. In contrast, the thalassemic sera show sharp decreases in fluorescence with increasing sample volumes. The concentration of NTBI in the sample is derived from the linear portion of the curve, and is quantified by use of a calibration curve, such as that shown in Fig. 1.

Example 4

Determination of Aluminium

The NTBI test was used for determining Aluminium (Al^{3+}) in solution and serum, since DFO has a high affinity for Al^{3+}, comparable to that for Fe. Solutions containing different concentrations of Al^{3+} were prepared by serial two-fold dilution in 20 mM Hepes-Na pH 7.3, of a stock solution containing 20 μM $AlCl_3$ in 20 mM Hepes-Na pH 7.3, with or without 10% normal human serum. A 0.1 ml sample from each solution was added to DFO-coated wells and the assay was carried out as described. Al^{3+} in solution is detectable in the NTBI test at concentrations above 0.2 μM, while Al^{3+} in 10% serum is detectable at concentrations above 1.25 μM. Thus, in full 100% serum, Al^{3+} has to reach 12.5 μM to become detectable in the assay.

The results of this experiment are shown in Fig. 3, wherein curve 1 relates to a solution containing 10% normal human serum and curve 2 relates to a solution containing no serum.

All the above description of procedures and preferred embodiments has been provided for the purpose of illustration, and is not intended to limit the invention in any way, except as defined by the appended claims. Many modifications can be provided in the various methods and materials. For instance, different metal ions can be detected, using different polymers and chelators, as well as different markers and coated surfaces, all without exceeding the scope of the invention.